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Summary

How social interactions influence cognition is a fundamental question, yet rarely addressed at the neurobiological level. It is well established that the presence of conspecifics affects learning and memory performance, but the neural basis of this process has only recently begun to be investigated. In the fruit fly *Drosophila melanogaster,* the presence of other flies improves retrieval of a long-lasting olfactory memory. Here, we demonstrate that this is a composite memory comprised of two distinct elements. One is an individual memory that depends on outputs from the α´β´ Kenyon cells (KCs) of the Mushroom Bodies (MBs), the memory center in the insect brain. The other is a group memory requiring output from the αβ KCs, a distinct sub-part of the MBs. We show that social facilitation of memory increases with group size and is triggered by CO_2 released by group members. Among the different known neurons carrying CO_2 information in the brain, we establish that the bilateral Ventral Projection Neuron (biVPN), which projects onto the MBs, is necessary for social facilitation. Moreover, we demonstrate that CO_2-evoked memory engages a serotoninergic pathway involving the Dorsal-Paired Medial neurons (DPM), revealing a new role for this pair of serotonergic neurons. Overall, we identified both the sensorial cue and the neural circuit (biVPN>αβ>DPM>αβ) governing social facilitation of memory in flies. This study provides the demonstration that being in a group recruits the expression of a cryptic memory and that variations in CO_2 concentration can affect cognitive processes in insects.

Keywords: drosophila, CO_2, social facilitation, memory, Kenyon cells, Dorsal-Paired Medial neurons, insect

Introduction

The ability of an individual to form distinct memories and refer to past experiences contributes to the survival of many species. Sensory stimuli from the environment are processed and integrated during memory formation and retrieval, sometimes impacting animal physiology over the very long term. In so-called "social" species, conspecifics are part of each individual's environment and constitute an important source of information that can lead to social learning [1–3]. While social learning has been widely examined in the literature, the influence of social context on memory retrieval has been poorly addressed, as most memory protocols are carried out on isolated individuals. This is not the case for the fruit fly *Drosophila melanogaster*, for which memory studies are generally carried out on groups and thus measure memory expression in a social context.

Despite a small brain of about 100,000 neurons, *Drosophila* can learn to associate and memorize different stimuli. A protocol leading to a measurable aversive olfactory memory is widely used in the literature. When exposed to one odor (conditioned stimulus plus, CS^+) associated with electric shocks *versus* another odor (conditioned stimulus minus, CS^-) without electric shock, flies learn the association between the CS^+ odor and electrical shocks and form an aversive associative olfactory memory. Memory is then scored using a T-maze offering a choice between two compartments enriched in the previously negatively reinforced CS^+ odor *versus* the non-reinforced CS^- odor [4] (Figure 1A). Memory is thus revealed by a selective avoidance of CS^+. After a single training protocol, this memory is short-lasting [4]. However, repeated training cycles generate a long-lasting memory which is measurable at least 24h after training. Multiple training cycles without any resting period (*i.e.* massed training) forms a consolidated memory that persists for at least 24 hours and is independent of *de novo* protein synthesis [5]. So far, this form of consolidated memory has been characterized as anesthesia-resistant memory (ARM) [5] since it is resistant to a cold-shock anesthesia [6]. Interestingly, memory after massed training is socially facilitated, as flies tested in groups perform better than individuals tested alone [7] which is not the case for short-lasting memory [8]. After massed training, only flies that express ARM are influenced by the social context during memory retrieval [7], which implies that ARM formed after massed conditioning is required to reveal this socially facilitated memory (hereafter “SFM”). Another form of consolidated memory can be generated by multiple training cycles performed with a 15 min resting period between each cycle (*i.e*. spaced training), which leads to a robust memory dependent at least partly on *de novo* protein synthesis and defined as long-term memory (LTM) [5]. A recent work proposed that spaced training leads to a dual memory composed of a safety memory for the CS^-, identified as the *de novo* protein synthesis LTM [9] and an aversive memory for the CS^+, which displays similarities with ARM generated by a massed training [9]. Unlike memory generated after massed conditioning, individual memory (i.e. memory performance of a fly tested alone) is much higher and not sensitive to the social context [7]. The lack of influence of the social context after spaced training could be explained by the high individual memory which would have reached a ceiling effect. Alternatively, the ARM generated by spaced conditioning might be different from that formed by massed training and not

be subject to SFM, or, although sharing similarities with ARM, the CS^+ memory measured after spaced training might not be ARM as formally described in other studies [7,10–13]. In any case, only memory formed after massed training is predisposed to SFM, for which memory performance increases in a social context. Although social facilitation of memory retrieval has been reported in humans [14], the increased memory performance of *Drosophila* tested in groups constitutes the first example of this phenomenon in invertebrates. Understanding the mechanisms underlying SFM could lead to insight into how social interactions influence cognition.

Results

Memory performance after massed training increases with the number of flies

We first investigated the influence of group size on memory retrieval. Groups of about 32 flies were subjected to massed trainings, and then different group sizes were tested 24h later in a T-maze. We found that memory performance increased with the number of trained flies tested together (1 to 32 individuals, Figure 1B). Interestingly, 24h after appetitive conditioning, where flies learned to associate one odor with a sucrose reward *vs*. another odor with no sucrose reward [15,16], flies tested alone or in groups obtained similar memory scores, confirming that individuals can achieve high memory performances irrespective of social context [8] (Figure S1). Appetitive training forms a long-term memory that depends on *de novo* protein synthesis, but it has not been clearly demonstrated whether appetitive training induces ARM or not [15–17]. This suggests that, in a general way, memory dependent of *de novo* protein synthesis leads to high individual memory which is not socially facilitated. 24h after massed aversive conditioning, where the social context has a positive influence on memory performances, we suspected that the cue inducing SFM may be a compound released by flies during stress, such as the previously reported *Drosophila Stress Odorant* (dSO), which contains CO_2 as its main component [18]. Our hypothesis was that trained flies exposed to aversive odorants would experience a stressful situation during memory testing and would release CO_2 (Figure 1C; Figure S2A). We thus investigated the contribution of CO_2 detection to SFM.

CO_2 exposure increases memory performance through biVPN activation

Using gas chromatography coupled to mass spectrometry (GC-MS), we assessed the amount of CO_2 released by groups of 4 (no SFM) or 32 (SFM) flies during odor exposures they would experience in memory testing (Figure 1D; Figure S2B). We observed that the difference between the levels of CO_2 released by groups of 4 and 30 individuals was greater than that measured between groups of 32 flies exposed or not exposed to CS^+. This result indicated that the number of flies, rather than the perception of odors previously associated to electric shocks (CS^+), would be the main factor driving the increase in CO_2 release. In our experimental conditions, flies experienced intense crowding in the elevator part of the T-maze just before the test (see Figure 1A, grey part of the T-maze). We examined whether this phase was critical for SFM expression. We found that groups of 4 flies (usually showing no SFM) reached higher memory scores when they

had been crowded within a larger group within the T-maze elevator (Figure S2C), meaning that pre-exposure to a large social group in the elevator was sufficient to enhance memory. This suggested that such increase in memory performance relied on the larger amount of CO_2 produced by a large group. Therefore, we predicted that exposing flies to CO_2 before the test should increase their performances. Based on the quantities of CO_2 measured with GC-MS (Figure 1D; Figure S2B), we tested memory performance following exposure of groups of 4 flies to an air flow enriched in 0.2%, 0.5% or 1% CO_2 for various amount of time (Figure 1E and 1F). Groups of 4 flies exposed to CO_2 immediately before the test showed better performance than flies exposed to normal air (Figure 1E and 1F), without impairing odor acuity (Figure S2D). As with the groups of 4 flies, single flies exposed to 1% CO_2 also showed improved memory (Figure S2E). These results show that CO_2 exposure before memory testing is sufficient to elicit increased memory performance.

Based on this finding, we then investigated the CO_2 neurons required for SFM. In flies, CO_2 primarily activates the V glomerulus of the antennal lobes, which is connected to higher-order brain structures by projection neurons called PNv1 (or biVPN), PNv2, PNv3 and PNv4 [19,20]. CO_2 exposure in naive flies did not elicit any disturbance of odor acuity (Figure S2C) while we found that blockade of PNv2 or PNv4 did (Figure S3A-S3D), suggesting that they are not directly engaged in the effect of CO_2 in SFM. We therefore focused primarily on biVPN and PNv3, since their blockade did not impair odor acuity (Figure S3E-S3G). We blocked synaptic transmission in biVPN (with the *R53A05-Gal4* [20] and *VT48643-Gal4* drivers [19]) and PNv3 (*VT12760-Gal4* driver [19]) during memory testing through expression of the dominant negative thermosensitive protein Shibire^{ts} (*UAS-Shi^{ts}*) [21]. While trained flies tested alone performed normally, blocking biVPN neurons, but not PNv3, altered the memory score of flies tested in groups of 32, hereafter "group test" (Figure 1G; Figure S3H and S3I). Flies with the same genotypes displayed normal memory at the permissive temperature for Shibire^{ts} (Figure S3J and S3K). This showed that biVPN neurons are necessary for CO_2-evoked SFM. By contrast, blocking biVPN activity 24h after spaced conditioning had no impact on memory performance (Figure S3L), demonstrating that the effects of CO_2 are specific to SFM. Our results further suggest that the nature of ARM generated by spaced training [9] differs from ARM formed after massed training or that spaced training does not generate ARM in the classical sense [10–13].

Mushroom bodies mediate both SFM and individual memory through distinct KCs

The CO_2-biVPN neurons project to the mushroom bodies (MBs), the main center of olfactory memory in *Drosophila* [22], and exposure to CO_2 induces an increase in MBs neuronal activity [20]. MBs are comprised of anatomically and functionally distinct neuronal populations called the αβ, α´β´and γ Kenyon cells (KCs) [6,10]. In order to identify the respective contribution of these neuronal populations to SFM, we silenced either outputs of all KCs (*VT30559-Gal4*) or, independently, the γ KCs (*NP21-Gal4*), the αβ KCs (*c739-Gal4* and *R44E04-Gal4)* or the α´β´ KCs (*G0050-Gal4* and *VT57244-Gal4)* during memory testing (Figure 2A-2D; Figure S4A-S4H). Blocking the output

of all types of KCs fully abolished memory retrieval (Figure 2A) without impairing odor acuity (Figure S4I). Blocking the output of αβ or α´β´ KCs, but not γ KCs (Figure 2B), impaired the performance of flies tested in groups (Figure 2C and 2D; Figure S4A-S4C) without impairing odor acuity (Figure S4J-S4M). Flies with the same genotypes displayed normal memory when tested at permissive temperature (Figure S4D-S4H). Interestingly, blocking αβ KC output specifically affected flies tested in groups (Figure 2C; Figure S4A), while the inactivation of the α´β´ KCs output also impaired memory in flies tested alone (Figure 2D; Figure S4B). Therefore, we conclude that the contribution of α´β´ KCs to memory retrieval is independent of the social context and that αβ KC output is required for SFM.

SFM and individual memory are independent co-expressed memories

Memory measured in groups 24h after a massed training is classically described as anesthesia-resistant memory (ARM), which is resistant to a cold-shock anesthesia and requires serotonin synthesis [5,23]. Since SFM and individual memory are processed differentially, we wondered whether they are separable memories that are co-expressed following massed training. Co-existing memories are known to be present 3-hours after one training cycle, when both ARM and a memory described as labile anesthesia-sensitive memory (ASM) are expressed [5]. Unlike ARM, ASM is cold-shock sensitive, serotonin-independent [11,23] and described as short-lasting [5]. We posited that ARM and ASM may also be co-expressed 24h after massed training, and correspond to the SFM and individual memories, respectively (Figure 3A-3C). To test this hypothesis, we blocked serotonin synthesis (Figure 3A) and performed cold-shock anesthesia (Figure 3B) on flies tested individually or in groups. Adult flies fed with para-chlorophenylalanine (pCPA), an inhibitor of serotonin synthesis, showed impaired group memory (Figure 3A). By contrast, cold-shock anesthesia affected both individual and group performances (Figure 3B). Thus, it appears that SFM is anesthesia-resistant and requires serotonin synthesis, while individual memory is anesthesia-sensitive and does not depend on serotonin synthesis. We next aimed to confirm these results by identifying the components of group memory that remained following selective inhibition of individual memory or SFM (Figure 3C). We found that blocking serotonin synthesis only impaired memory remaining after the blockade of α´β´ KCs (SFM) and that cold shocks specifically affected the memory remaining after blocking αβ KCs (individual memory). Therefore, we conclude that massed training leads to both ASM (individual memory) and ARM (SFM), and that the latter component is only expressed in a group setting. These two memories are qualitatively different, processed in different neuronal subsets and co-expressed during 24h memory retrieval.

SFM requires 5HT1A activity in αβ KCs

As αβ KCs and serotonin signalling are required for normal SFM expression (Figure 3C), we then aimed to identify the serotonin receptor in the αβ KCs involved in SFM. We used RNAi to selectively knock down the expression of the 5HT1A or 5HT1B receptors [24,25], two serotonin receptors known to be expressed in *Drosophila* KCs [26], in the αβ KCs of adult flies (*tub-Gal80^{ts}; c739-Gal4*) [27]. Memory performance of flies tested in groups was impaired when 5HT1A receptors,

but not 5HT1B, were knocked down (Figure 4A; Figure S5A-5C), indicating that SFM requires 5HT1A serotonin receptor activation in the αβ KCs.

Serotonin from DPM neurons is necessary for SFM

To identify the serotoninergic neurons involved in SFM, we first investigated a large number of serotoninergic neurons marked by the *Ddc-Gal4* driver [28]. Inhibiting Ddc neurons, which impairs place memory (another form of associative learning [29]), did not affect memory retrieval in groups (Figure S5D). We then investigated a pair of serotoninergic neurons, the Dorsal Paired Medial neurons (DPM), which are not labeled by the *Ddc-Gal4* driver [23]. These neurons are known to be involved in short-lasting ARM consolidation [23] but their role in long-lasting ARM retrieval has not yet been examined. Silencing DPM during memory testing (*VT064246-Gal4>UAS-Shi^{ts}* flies) impaired group but not individual memory performance (Figure 4B; Figure S5E and S5F), showing a specific role in SFM. Because DPM produce both serotonin and gamma-amino butyric acid (GABA) [30], we expressed RNAi against the enzymes catalysing synthesis of these two neurotransmitters, specifically at the adult stage (Figure 4C and 4D; Figure S5G and S5H). Only lowering serotonin levels decreased memory performance in flies tested in groups but not alone (Figure 4C), confirming that serotonin from DPM neurons is indeed specifically necessary for SFM. We conclude that the expression of SFM requires release of serotonin from DPM neurons, which signals through 5HT1A receptors in αβ KCs.

CO_2 modulates odor-evoked responses in DPM neurons

Consistent with the central role for CO_2 in triggering SFM, we found that an exposure to 1% of CO_2 no longer improved memory performance in groups of 4 flies with impaired DPM activity (*VT064246-Gal4>UAS-Shi^{ts}*) (Figure 5A). Moreover, using an *in vivo* imaging protocol with a calcium reporter (*UAS-GCaMP6f*) specifically expressed in DPM neurons (*VT064246-Gal4*), we observed that DPM response to odors was modulated by CO_2. We recorded DPM activity in trained flies exposed to CS^+ and CS^- odors, before and after a 30s exposure to 1% CO_2 (Figure 5B and 5C; Figure S6A-S6G), a condition sufficient to elicit SFM. We found that DPM were significantly less responsive to CS^- after flies have been previously exposed to CO_2 (Figure S6E). Such decrease in the response to the CS^- increased the response ratio of CS^+/CS^-, augmenting the relative prominence of the CS^+ (Figure 5C; Figure S6A and S6B). Flies exposed to a pure air flow did not show such modulation in DPM response to CS^- (Figure S6G) and the CS^+/CS^- response ratio remained constant over time (Figure 5C; Figure S6A and S6C).

biVPN and DPM neurons communicate through mushroom bodies KCs

Finally, we sought to define the neuronal pathway from detection of CO_2 to the expression of SFM. The biVPN neurons are known to project onto the MB calyx [19,20] and the DPM neurons have been shown to project to all lobes of the MBs [31]. Interestingly, DPM and MBs establish contacts in a bidirectional way as indicated by recent evidence of connections from α KCs to DPM neurons [32]. A direct anatomical link between biVPN and DPM is unlikely given that these neurons do not project on the same MB areas [19,31]. We used GFP Reconstruction Across Synaptic Partners

(GRASP), a tool employed to identify synaptic contacts (Figure S6H) [33,34], to confirm that there is no direct anatomical contact between biVPN and DPM (Figure S6I-S6K). This suggests that CO_2 information conveyed by the biVPN reaches the DPM indirectly *via* the activation of MBs neurons. Thus, we propose that SFM likely depends on a "biVPN - αβ KCs - DPM - αβ KCs" pathway (Figure 6). Above a certain threshold, CO_2 released by flies during memory retrieval activates biVPN neurons, triggering MB neurons that recruit DPM. In turn, DPM modulate the activity of αβ KCs *via* serotonin.

Discussion

We showed that CO_2 can act as a facilitating cue leading to an improvement in memory retrieval. Moreover, we demonstrated that such improvement relies on the expression of ARM formed after a massed training, which is expressed distinctly from individual memory, and we identified the neural network supporting the expression of this additional CO_2-sensitive memory. We showed that memory retrieval within a group relies on the recruitment of a second neural network in addition to the one required when flies are tested alone. SFM is not a simple improvement of the expression of an individual memory but constitutes a memory expression in its own right. Therefore, the memory revealed in a social context is actually a composite memory consisting of two previously encoded memories, ASM and ARM, whose expression relies on distinct neural structures. Expression of these memories are indeed independent and additive given that the inhibition of one memory during the retrieval phase does not impair the expression of the other. Thus, this work has provided evidence that ASM is the memory expressed when flies are tested individually and is independent of CO_2, while SFM has been characterized as the additional expression of ARM in a social context.

The predictability of a US by an originally neutral stimulus becomes higher upon repetition of the stimulus pairing over extended periods. In *Drosophila*, two types of aversive long-lasting memories have been characterized. On the one hand, the composite memory described in the present study which arises after massed training and is independent of protein synthesis [5]. On the other hand, another form of consolidated memory that occurs after spaced training [5] and which is dependent on *de novo* protein synthesis (LTM) [5,9]. Recently, this consolidated memory has been defined as the addition of LTM and ARM, an aversive memory independent of protein synthesis [9]. ARM potentially generated by spaced training and the socially facilitated ARM generated by massed training would involve distinct molecular processes, as suggested by the distinct pathways recruited by spaced and massed trainings. Indeed, pCPA treatment [11,12,23], the *Drk* mutation [35] or the biVPN blockade (this study) impairs the memory formed after massed training but not the memory generated by spaced training. Like ARM measured after a massed conditioning, the CS^+ memory measured after spaced training is Radish-dependent which led to its characterization as ARM [9].

However, the memory generated by spaced conditioning does not seem to share the other ARM characteristics detailed above and it should be considered that this CS^+ memory would not be ARM in the classical sense, as supported by other studies [10–13,36]. In any case, memory formed after spaced training is the most stable form of memory reported in *Drosophila* and can last up to 7 days post-training. It enables high individual retrieval performances [7] but requires, at least in part, *de novo* protein synthesis (LTM) [5,9,11] involving metabolically costly processes [37], which can occur at the expense of an animal's fitness under stressful conditions [12,38]. Similarly to aversive LTM formed after spaced training, long-lasting appetitive memory depends on *de novo* protein synthesis [15,16]. Interestingly neither aversive or appetitive memory dependent on protein synthesis is socially facilitated. SFM mechanism, purely independent of protein synthesis, would then allow flies to behave appropriately while reducing the costs of learning. Surprisingly, social context does not influence the formation of SFM, but rather only its retrieval [7]. This suggests that CO_2 possibly released by flies during training does not foster individual learning, which would indicate that the training procedure used in our study generated sufficiently high levels of learning for the influence of the social context to become negligible. As CO_2 is not necessary for the retrieval of memory formed after aversive spaced training, we conclude that CO_2 does not play a general role as a memory enhancer. This aspect deserves further investigation.

Besides *Drosophila*, an influence of the social context on memory retrieval has been highlighted in humans, first addressed by Kenneth Spence in 1956 and summarized by the Drive theory [14]. According to this theory, an individual's performance is potentiated by the presence of other individuals provided that the task performed has been correctly learned beforehand. Social facilitation of memory in *Drosophila* is consistent with this theory. Yet, as the studies in humans have focused only on short-term restitution, the influence of social context on long-lasting retrieval evinced in our work remains to be addressed in other taxa, such as rodent or insects. Memory tests are typically conducted on individuals as the characterization of memory refers to an individual's acquisition, storage and retrieval of information. Yet, in the light of our findings, it would be interesting to determine to what extent social context affects memory retrieval in other animal species.

Here, we showed that CO_2 recruits additional circuits leading to the socially facilitated ARM expression. Flies emit and process more CO_2 in a group, possibly integrating CO_2 as a marker of stress [18]. Therefore, CO_2 can be conceived as a stress cue enhancing a fly's attention, changing its representation of the environment, and mediating the expression of an additive memory. Indeed, we have provided evidence that exposure to CO_2 alters the CS^- response in DPM neurons, which could stimulate fly's awareness to the CS^+ memory trace by inhibiting the responses to the irrelevant CS^- stimulus. In vertebrates, moderate stress can promote aversive long-lasting memory [39,40]. Although memory mechanisms described for vertebrates differ from those in our model, the benefits of moderate stress on memory seem to be common across species.

So far, the role of CO_2 in insect behavior has been mostly limited to naive avoidance and attraction [18,41–47]. Here, we reveal an important role for CO_2 as a facilitator of olfactory memory. In natural environments, CO_2 is a ubiquitous cue, including within the nest of eusocial insects such as ants, termites or bees [48], that can be potentially significant and attractive. It is an attractive cue for insects at food sources and oviposition sites [43,49] and also plays a key role in host detection for hematophagous insects such as the tsetse flies [50,51] or mosquitoes [52]. Olfactory learning plays a significant role in host preference and disease transmission in blood-feeding insects [53]. Thus, exploring the impact of CO_2 on memory processes in these insects would be interesting to develop and improve control strategies to reduce the risk of disease transmission. Our findings suggest that CO_2, may have an unsuspected impact on the cognition of a broad spectrum of insect species.

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Author contributions

A.M. performed all the behavioural experiments in this study. P-Y.M. performed the GRASP experiments in the M.D.G. lab. A.M. and M.D. performed all imaging experiments with B.R. and P-Y.M. contribution. B.R., A.M., P-Y.M. and G.I. devised a new olfactory stimulation device under the microscope. A.M. performed all the GC-MS experiments under F.R.P supervision. R.J. developed a R code software to analyse *in vivo* imaging data. A.M., R.J., and G.I. wrote the paper with P-Y.M., M.D. and M.D.G. proofreading; G.I. supervised the project and designed all experiments with A.M.

Declaration of interests

The authors declare no competing interests.

Main-text figure legends:

Figure 1. CO_2 improves memory retrieval through biVPN neurons activity. (A) Experimental protocol. (B) Memory retrieval performance after massed training increases with number of flies tested (n = 12). (C) Hypothesis: the amount of CO_2 released increases with the number of flies, leading to SFM expression when integrated CO_2 is above a threshold. (D) Amount of CO_2 detected by GC-MS (see Methods), released by groups of 4 or 30 flies in the absence of odorant (n = 22 for groups of 4 flies and n = 24 for groups of 30 flies) or exposed to the odors CS^- (*green, n* = 30 for groups of 4 and 30 flies) or CS^+ (*orange n* = 30 for groups of 4 flies and n = 29 for groups of 30 flies). The percentage of CO_2 released has been calculated for each condition. (E) Memory performance of trained groups of 4 flies increases after exposure to 1% of CO_2 for 30s (n = 8). Control groups have not been exposed to any stimulation (n = 12). (F) Memory performances of groups of 4 flies exposed for 20s, 30s, 60s, 90s or 180s to either 0.2%, 0.5%, 1% of CO_2 or to a pure air flow, just before memory test (n = 8). (G) Temporal blocking of biVPN CO_2-sensitive neurons (*R53A05-Gal4>UAS-Shi^{ts}* flies) during individual or group (32 flies) memory test (n = 10). For data in (B), Tuckey's multiple comparisons of means, different letters indicate a significant statistical difference between groups. For data in (D), (E) and (G), Tukey's multiple comparisons of means, **p <0.01, ***p <0.001. For data in (F), *t*-test, memory scores following CO_2 exposure were compared to the corresponding air flow control (same exposure time), ****p <0,0001. Data are represented as mean ± SEM. See also Figures S1-S3.

Figure 2. Mushroom bodies are necessary for both individual memory - through the α´β´ KCs - and the group memory leading to SFM - through the αβ KCs. (A) Temporal blocking during the memory test of the whole KCs (*VT30559-Gal4>UAS-Shi^{ts}, n* = 12 for each condition), (B) the γ (*NP21-Gal4>UAS-Shi^{ts}, n* = 12 for each condition), (C) the αβ KCs (*c739-Gal4>UAS-Shi^{ts}, n* = 13 for each group condition and n = 8 for each individual condition) and (D) the α´β´ KCs (*G0050-Gal4>UAS-Shi^{ts}*, n = 8 for each condition) outputs. For all data, Tukey's multiple comparisons of means, *p <0.05, **p <0.01, ***p <0.001. Data are represented as mean ± SEM. See also Figure S4.

Figure 3. αβ KCs activity leading to SFM is dependent on serotonin synthesis while α´β´ KCs activity leading to individual memory is cold-shock sensitive. (A) Individual and group retrieval performances of flies fed with an inhibitor of serotonin synthesis (pCPA, n = 10 for group and individual test) or with a sucrose solution (control, n = 10 for group and individual test). (B) Cold-shock anesthesia (see Methods) in trained flies tested alone or in group (n = 12 for each condition). (C) Inhibition of serotonin synthesis (pCPA) or/and cold shock anesthesia (2 min at 0°C) in wild-type flies, or in flies with a temporal blockade of αβ KCs (*c739-Gal4>UAS-Shi^{ts}*) or α´β´ KCs (*G0050-Gal4>UAS-Shi^{ts}*) outputs during memory test in group (n = 10 for each condition). For data in (A), (B), *t*-test, *p <0.05, **p <0.01. For data in (C), Tukey's multiple comparison of means, *p <0.05, **p <0.01. Data are represented as mean ± SEM.

Figure 4. Serotonergic signaling from DPM neurons is necessary for the anesthesia-resistant SFM through 5HT1A serotonin receptor in the αβ KCs. (A) Temporal downregulation of 5HT1A serotonin receptor in the αβ KCs specifically at adult stage (Heat induction, see Methods) (*c739-Gal4; Tub-Gal80^{ts}/+>UAS-RNAi 5HT1A*, $n = 12$ for each condition). (B) Temporal blocking of DPM neurons (*VT064246-Gal4>UAS-Shi^{ts}*) output during memory test ($n = 12$ for each condition). (C) Temporal down expression at adult stage of the enzyme responsible for serotonin synthesis (ddc, $n = 10$ for each condition) or (D) GABA production (GAD, $n = 8$ for each condition) in DPM neurons (Heat induction), in respectively *Tub-Gal80^{ts}*; *VT064246-Gal4 > UAS-RNAi ddc* and *Tub-Gal80^{ts}/+; VT064246-Gal4>UAS-RNAi GAD* flies. For all data, Tukey's multiple comparison of means, **p <0.01, ***p <0,001. Data are represented as mean ± SEM. See also Figure S5.

Figure 5. CO_2, mushroom bodies and DPM neurons are acting together for SFM. (A) Groups of 4 flies exposed to 1% of CO_2 for 30s right before memory test, while DPM neurons output are temporally blocked during the test (*VT064246-Gal4>UAS-Shi^{ts}*, $n = 10$ for each condition). (B) *In vivo* imaging protocol performed in flies expressing the calcium reporter *UAS-GCaMP6f* in DPM neurons (*VT064246-Gal4*). Visualization of DPM neurons projections on mushroom bodies median neurons in response of either the CS^+ or the CS^- odor, before, just after exposure (immediate) and 1min, 2min and 3min after exposure to a pure air flow or to 1% of CO_2. (C) Ratio between the response of DPM neurons to the odors CS^+ and CS^- ($\Delta f(CS^+)/\Delta f(CS^-)$) in *VT064246-Gal4>UAS-GCaMPp6f* flies, before and after exposure to an air flow enriched in 1% of CO_2 (immediate, 1min after, 2min after and 3min after, $n = 10$) or to a pure air flow ($n = 8$). For data in (A), *t*-test, *p <0.05, ***p <0.001. For data in (C) *t*-test, *p <0.05 when compared to the air flow condition. Data are represented as mean ± SEM. See also Figure S6.

Figure 6. A model for social facilitation of memory retrieval. During group test 24 hours after a massed aversive olfactory conditioning, two memories are co-expressed: CO_2-independent individual memory (ASM) requiring α´β´ KCs activity and CO_2-dependent group memory (SFM/ARM) requiring biVPN neurons and αβ KCs /DPM neurons loop activity.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Guillaume Isabel (guillaume.isabel@univ-tlse3.fr).

Materials Availability Statement

The programming details of the automated olfactometer prototype generated by this study are legally protected through registered software and are confidential. For any information about licensing and potential exploitation, please contact our TTO sante@toulouse-tech-transfer.com.

Data and Code Availability Statement

The datasets generated during this study are available at Mendeley Data (DOI: 10.17632/gnr8xws4cr.1)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We conducted all experiments using 2-6 days old male and female *Drosophila melanogaster*. We bred flies in incubators on a 12 hours light: 12 hours dark cycle at 25°C or 18°C depending on the experiment, on standard yeast and cornmeal fly food.

METHOD DETAILS

Fly strains

Fly stocks were maintained on standard corn meal/yeast/agar medium at 25°C. The fly lines used were wild-type Canton S w1118, *tubulinGAL80^{ts}*, *UAS-GCaMP6f* [54], *UAS-Shi^{ts}* [21], *UAS-RNAi 5HT1A (VDRC 106094), UAS-RNAi 5HT1B (VDRC 9558), UAS-RNAi ddc (VDRC 3329), UAS-RNAi GAD (VDRC 32344), Ddc-gal4 neurons (Bloomington 7009)*, DPM (*VT064246-GAL4 and L0111-LexA*), PNv2 (*NP7273-GAL4*), biVPN (*R53A05-GAL4* and *VT048643-GAL4*), PNv3 (*VT012760-GAL4*), PNv4 (*E0564-GAL4*), αβ (*c739-GAL4* and *R44E04-GAL4*), α'β' (*G0050-GAL4* and *VT057244-GAL4*), γ (*NP0021-GAL4*) and MB (*VT030559-GAL4* and *R26E07-LexA*). For GRASP experiments, we used the following constructions: *UAS-CD4::spGFP1-10* and *LexAop-CD4::spGFP11*.

Starvation protocol

Before training, 2-day-old wild type Canton S flies raised at 18°C were kept in groups of 30 flies in plastic bottles containing a cotton pad imbibed with 6 ml of mineral water (pH = 7.2; Evian®) at 25°C and 60% of humidity for 21h.

Sucrose delivery

24h before training, a 1.5 M sucrose solution diluted in mineral water (Evian) was applied on 2/5 of the inner surface of plastic tubes, using a cotton pad imbibed with 1ml sucrose solution. The sucrose tubes were left to dry at room temperature.

Olfactory training

We performed a classical associative discriminative olfactory conditioning protocol [5,16] on 2-day-old flies (aversive training) or 3-day-old flies (appetitive training) using two well discriminated odors: 3-octanol (OCT, 2.27mM) and 4-methylcyclohexanol (MCH, 2.62mM). During training, flies were successively exposed to the two odors carried through the training chamber in a current air flow (400mL/min/training chamber). A cycle of conditioning consisted of 90s of pure air before exposing flies to the conditioned stimulus (CS^+) and the unconditioned stimulus (US) simultaneously for 60s, then, the chamber was cleaned with fresh air for 45s before exposing the flies to the CS^-, which was not paired with the US. The US consisted of twelve 1.5s pulses of 60V electric shock every 5s for aversive conditioning and in rotating the barrels to expose sucrose applied on plastic tubes for appetitive conditioning. For 24-hour memory experiments, flies were subjected to a single training cycle (appetitive training), five training sessions in a row (aversive massed training) or spaced out with a 15 min rest interval (aversive spaced training). Each experiment was performed on flies conditioned either with the odor OCT as CS^+ or odor MCH as CS^+. All trainings were performed on groups of approximately 30 flies, at 25°C and 70% relative humidity.

After conditioning, flies were maintained for the night on standard medium at 18°C, following aversive trainings, or in plastic bottles containing a cotton pad imbibed with 4ml of mineral water (Evian), following appetitive training.

Memory test

24h after training, flies trained together were transferred in a T-maze comprising two phases: phase 1, where the flies were "confined" in the upper tube and in the elevator, and phase 2, where the flies faced a choice between two lateral compartments filled by an air flow carrying either OCT or MCH (400 mL/min/compartment). They were allowed to choose between the CS^+ and the CS^- for 3 min, at which time they were trapped inside their respective compartments. Flies that remained in the center of the T maze and did not choose a compartment were excluded from the analysis. The test was carried out in a climate room at 25°C (for experiments using CO_2) or 33°C (for experiments using the Shibire dominant-negative tool) and 70% relative humidity, under red light (OSRAM 64543; 230V, 42W bulb covered with a red filter paper Rosco E-Fire #19). Flies were tested individually or by group of 2, 4 (hereafter, small group), 8, 16 or 30-32 flies (hereafter, large group) depending on the experiment. For the tests with single flies and small groups, individuals were trained in a large group and then isolated from the others one hour before memory test. Flies tested in large group were never isolated.

To evaluate the influence of crowding during phase 1 of the T-maze protocol on memory performances, we introduced groups of either 4 or 30 flies during 30s in the T-maze elevator. Afterwards, we tested either the 4 flies that were by groups of 4 in the T-maze or 4 flies that have been randomly sampled in the groups of 30 flies. The performances of groups of 30 flies was also scored.

Performance index

We calculated a performance index (PI) to score the memory of conditioned flies. For appetitive memory, the index is given by the number of flies in the CS^+ compartment minus the number of flies in the CS^- compartment divided by the total number of flies in the two compartments. In the opposite, for aversive memory, the index is given by the number of flies in the CS^- compartment minus the number of flies in the CS^+ compartment divided by the total number of flies in the two compartments. Since we were testing different group sizes, it was necessary that the memory score of each replicate, regardless of group size, be based on an equivalent number of individuals. To this end, we proceeded as follows.

- For groups of 8, 16 or 32 flies, one replicate consisted in testing independently one group of flies conditioned with OCT as CS^+ and one group of flies conditioned with MCH as CS^+. The PI of each replicate consisted in averaging the scores obtained in the 2 tests.
- For groups of 4 flies, one replicate consisted in testing independently 2 groups of flies with OCT as CS^+ and 2 groups of flies with MCH as CS^+. The PI of each replicate consisted in averaging the scores obtained in the 4 tests.
- For groups of 2 flies, one replicate consisted in testing independently 4 groups of flies with OCT as CS^+ and 4 groups of flies with MCH as CS^+. The PI of each replicate consisted in averaging the scores obtained in the 8 tests.
- For single flies, one replicate consisted in testing independently 8 flies with OCT as CS^+ and 8 single flies for MCH/CS^+ [7]. The PI of each replicate consisted in averaging the scores obtained in the 32 tests.

A total of 7 to 12 replicates were performed for each condition.

CO_2 behavioral experiments

Groups of 4 flies were exposed to either a pure or "CO_2 enriched" air flow (800 mL/min) in the upper part of the T-maze. The exposure time varied (20s, 30s, 60s, 90s and 180s), depending on CO_2 concentration (0.2%, 0.5%, 1% and 5%) which was controlled by an air/CO_2 mixer (CO_2 controller, PeCon). Immediately after CO_2 exposure, the flies were introduced to the T-maze point choice and were submitted to the memory test.

Olfactory acuity

We assessed olfactory acuity by introducing groups of approximately 30 naive flies to the T maze. Each group of flies was given a choice between one arm enriched in one odor (OCT or MCH) and one arm with pure air for 3 min. We then computed an avoidance index given by the number of flies in the "no odor" compartment minus the number of flies in the "odor" compartment divided

by the sum of flies in the two compartments. We performed 12 replicates for each odor.

pCPA experiments

Flies bred at 18°C were exposed to pCPA according to the protocol described in Plaçais et al., 2012 [11].

Cold-Shock experiments

1 hour before testing, trained flies were exposed to a cold shock (0°C) for 2min.

RNAi experiments

Expression of RNAi(s) was induced by exposing flies at 30°C for 5 days before training (heat induction). Control groups have been exposed at 18°C for 5 days (no heat induction).

***In vivo* calcium imaging**

2-day-old transgenic flies which expressed the *UAS-GCaMP6f* calcium probe were subjected to a massed training. 24 hours later, they were dissected according to the protocol described in Fiala and Spall [54] and then imaged under a confocal microscope (Leica TCS SP5) equipped with a water immersion objective (25X NA 0.95). Argon laser was set to a 400Hz in a bidirectional mode. Calcium probe was excited at 488nm wavelength and signal was detected at 505-555nm. Images (format at 512*256 pixels) were acquired at a rate of one image every 400ms. To visualize the neural structures, pinhole was open at 300µm. Flies were exposed to odors CS^+ and CS^- (OCT, 21.86mM and MCH, 24.97mM) and to an air flow enriched or not with 1% of CO_2, following a cycle previously programmed with an automated olfactometer prototype based on an Arduino microcontroller and coupled (triggered by TTL) to the microscope's scanning head, allowing real-time synchronization between image acquisition and olfactory stimulation. The programming details of the automated olfactometer prototype generated by this study are legally protected through registered software and are confidential. For any information about licensing and potential exploitation, please contact our TTO sante@toulouse-tech-transfer.com.
After 20s of baseline the CS^+ and CS^- odors were delivered for 5s with a 15s break between the two odors. 15s after a first stimulation couple CS^+/CS^-, flies were exposed for 30s to a pure air flow or enriched with 1% CO_2. 10s after air or CO_2 exposure, the CS^+ and CS^- odors were delivered again (immediate) for 5s with a 15s break between the stimulations. The CS^+/CS^- couple was released again 1min, 2min and 3min post-exposure with a 25s break between two CS^+/CS^- stimulations. The CS^+/CS^- sending order was balanced between flies. The baseline was monitored for 20s before sending any odorant stimulation. After registration, images were collected and a standardized region of interest (ROI) was centred within the DPM projection onto the MB ββ' lobes area. Analysis was performed with Fiji/ImageJ (RRID: SCR_001935) and intensity tables were exported to the R 3.2.2 software (RRID: SCR_001905) and the ΔF/F was calculated for each stimulation. The basal fluorescence "F" was the averaged 20 images preceding an odorant stimulation. Then, the ΔF/F intensities were exported to Excel and the ratio $\Delta F/F_{CS+}$/ $\Delta F/F_{CS-}$ were calculated for each stimulation time (-1min$_i$, immediate, 1min, 2min and 3min). The ratio $\Delta F/F_{CS+}$

/ $\Delta F/F_{CS-}$ of flies which were subjected to a pure air flow were compared to the ratio computed for flies that received a "CO_2 enriched" air flow.

Gas chromatography and mass spectrometry

Wild type flies were submitted to a massed training and maintained at 18°C overnight. 24h after training they were exposed to the odors CS^+ or CS^- in an auto-sampler tube of 10ml for 3min and then immediately frozen in a solution of liquid nitrogen. The CS^+ or CS^- odors were diluted in paraffin oil (OCT, 0.50mM and MCH, 0.53mM) and 1µl of solution was pipetted on a Whatman paper in the chromatography tube. Air samples from tubes containing CO_2 were analysed using a mass spectrometer quadrupole detector (ISQ QD) coupled to a Trace 1300 gas chromatography (Thermo Fisher Scientific Inc., Illkrich, France), fitted with a capillary column (Restek RTX-5MS 30 m×0.25 mm, 0.25µm film thickness, 5 % diphenyl and 95 % dimethylpolysiloxane) and a splitless injector (270 °C). Helium was the carrier gas (1.2 mL/min). The oven temperature was maintained at 70°C. The operating conditions for the MS were 10 to 100 m/z, 9.6 scans/seconds and ionisation by electron impact (70 eV, source temperature 250 °C). 20 µL of air from each tube were injected with a Hamilton syringe into the GC column. For identification of CO_2 a selected ion monitoring at m/z=44 Dalton was carried out.

GRASP experiments

Immunohistochemistry was carried out as described previously [55]. The primary antibodies used were mouse anti-GFP (1:100, Sigma Catalog #G6539) and mouse anti-nc82 (1:50, Developmental Studies Hybridoma Bank). The secondary antibodies used were goat anti-rabbit Alexa Fluor 488 (Invitrogen, #A11008), goat anti-mouse Alexa Fluor 568 (Invitrogen, #A11036). Images were maximum intensity projections of confocal z stacks acquired using a Leica SP5 II confocal microscope with the 25X water immersion objective.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analyses were performed using the R 3.2.2 software. Data normality and homoscedasticity have been checked with Shapiro-Wilk and Levene tests, respectively. The different conditions (genetic and experimental) were compared using ANOVA followed by post-hoc Tukey tests. Kruskal-Wallis tests followed by post-hoc Dunn tests were used when conditions of normality and homoscedasticity were not met. We used *t*-tests to compare at each stimulation time (-1min, immediate, 1min, 2min and 3min) the ratios ($\Delta FCS^+/Fi$) / ($\Delta FCS^-/Fi$) between flies from the "air" and "CO_2" groups. In all groups, learning performance was assessed by memory score to 0 (chance) with a Wilcoxon test.

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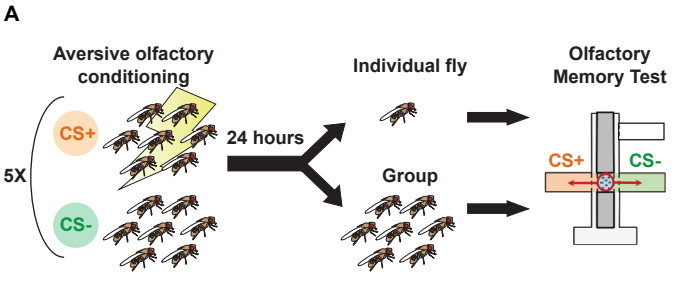
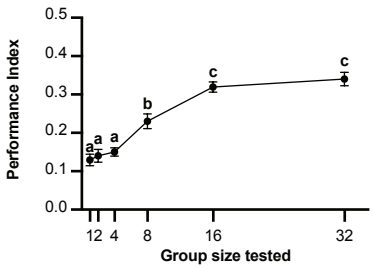
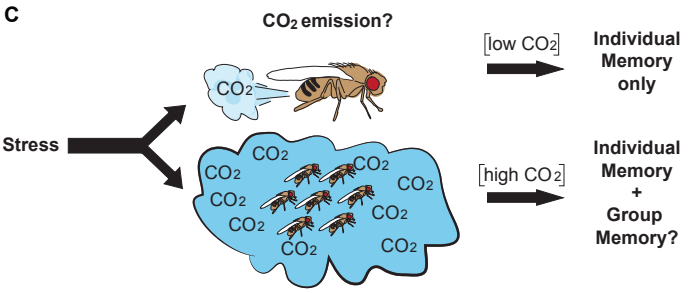
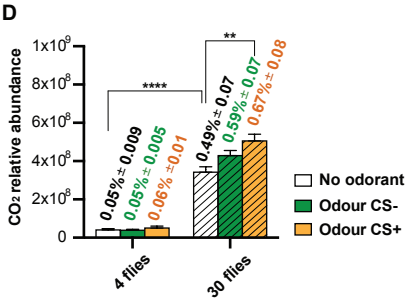
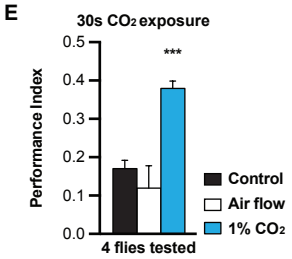








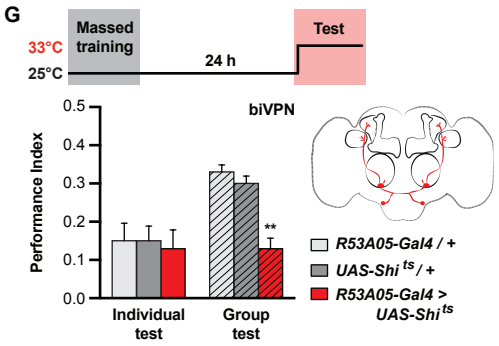


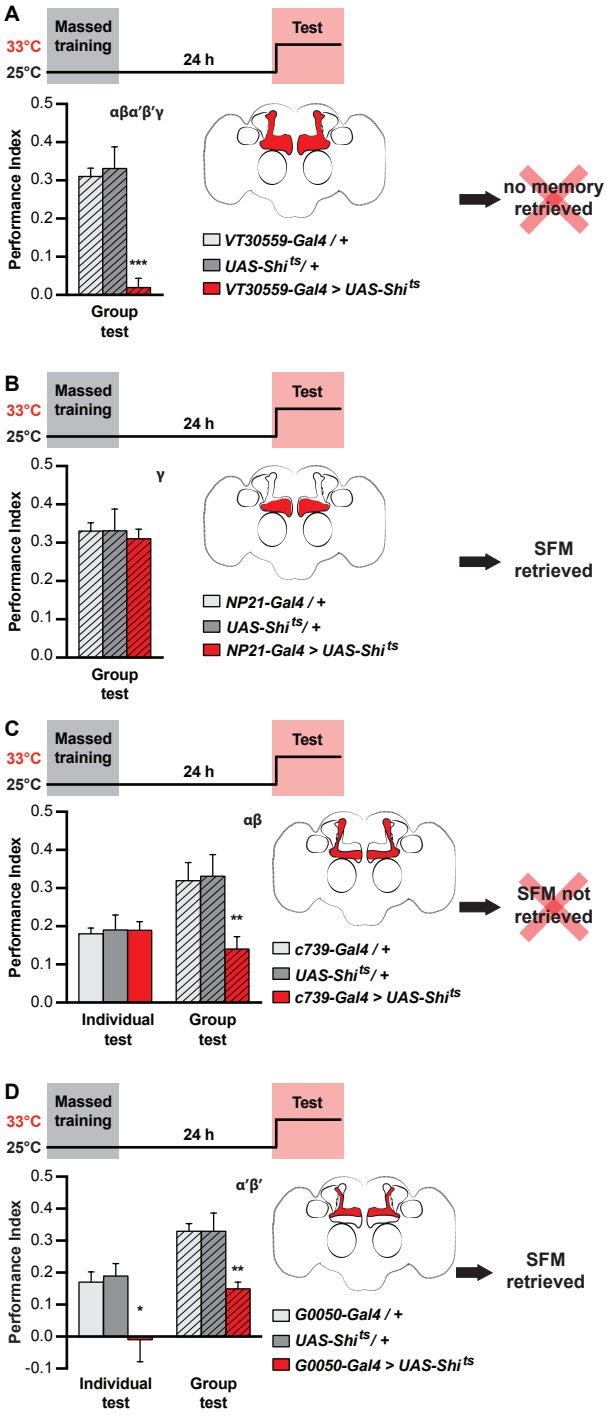
F

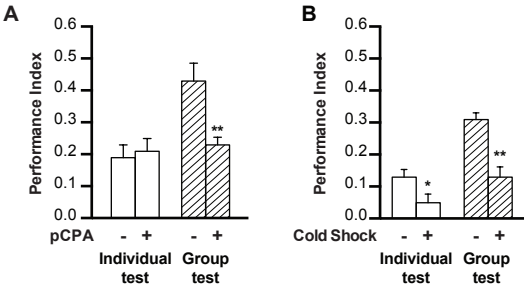
Exposure time	Air flow	0.2% CO_2	0.5% CO_2	1% CO_2
20s	0.13 ± 0.060			0.17 ± 0.022
30s	0.11 ± 0.038			0.36 ± 0.023 ***
60s	0.12 ± 0.058		0.17 ± 0.023	0.38 ± 0.019 ***
90s	0.14 ± 0.051	0.16 ± 0.022	0.37 ± 0.018 ***	
180s	0.15 ± 0.047	0.41 ± 0.035 ***		

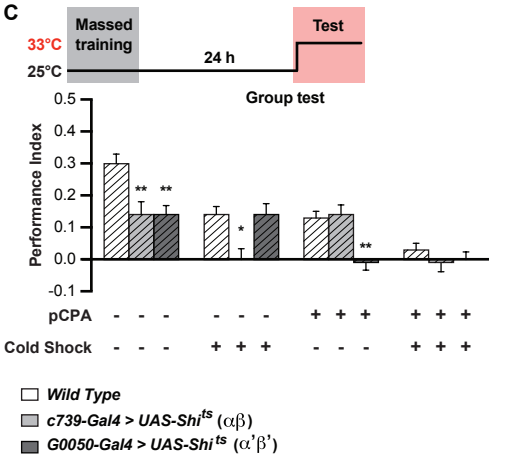
CO_2 concentration

***: significantly different from Air flow control

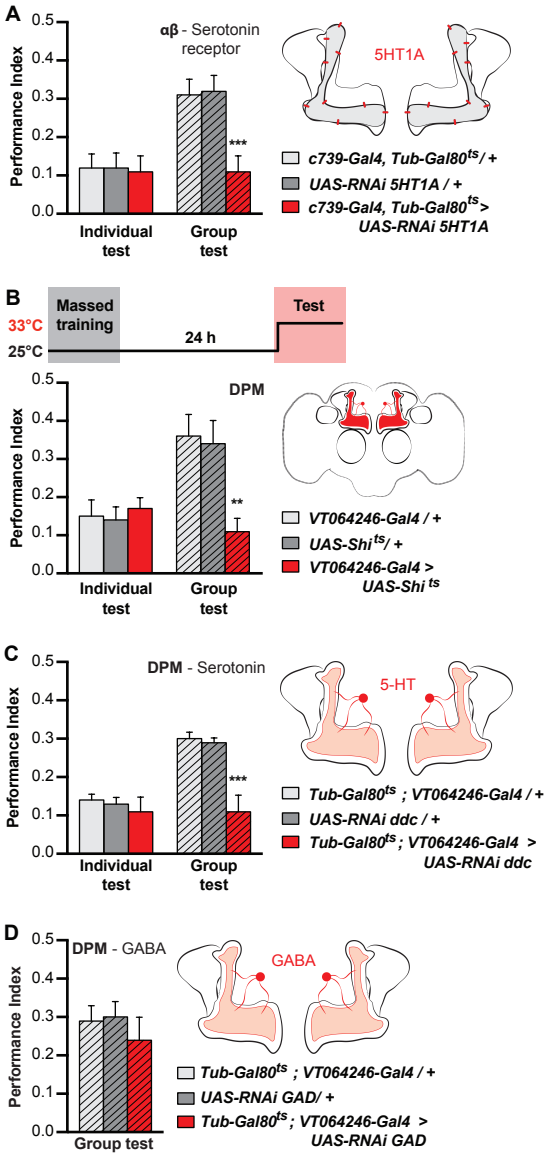


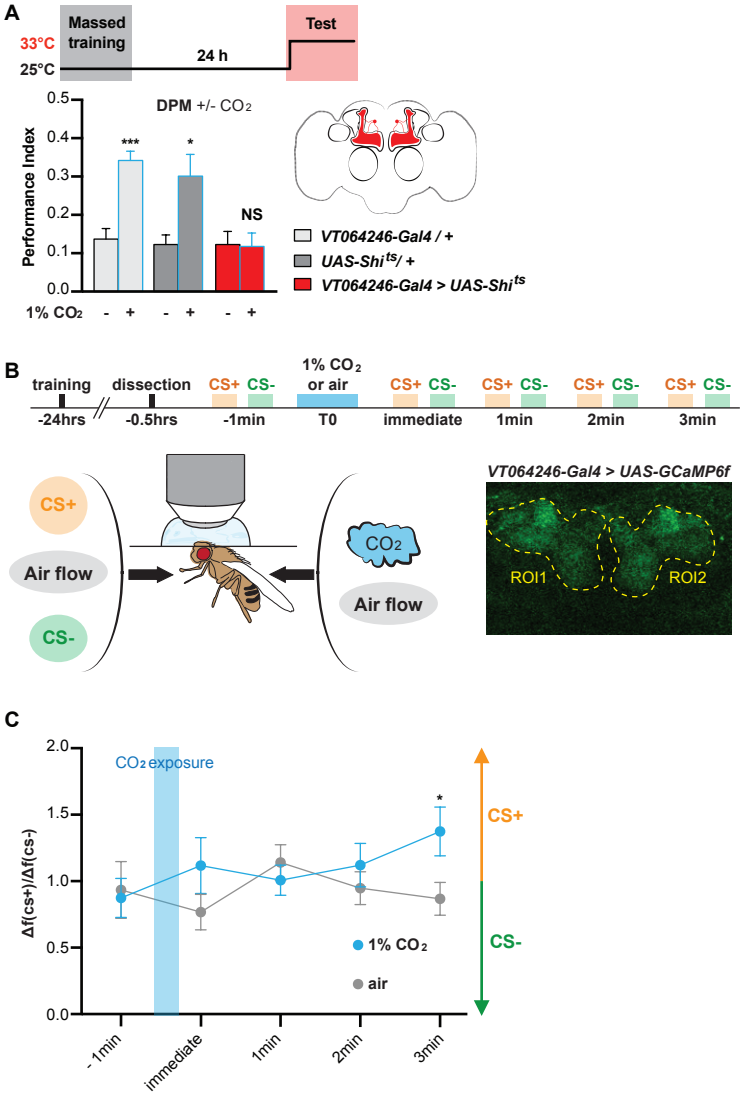

A
Massed training
Test
33°C
25°C
24 h
αβα′β′γ
Performance Index
0.5
0.4
0.3
0.2
0.1
0.0
***
Group test
VT30559-Gal4 / +
UAS-Shi ts / +
VT30559-Gal4 > UAS-Shi ts
no memory retrieved
B
Massed training
Test
33°C
25°C
24 h
γ
Performance Index
0.5
0.4
0.3
0.2
0.1
0.0
Group test
NP21-Gal4 / +
UAS-Shi ts / +
NP21-Gal4 > UAS-Shi ts
SFM retrieved
C
Massed training
Test
33°C
25°C
24 h
αβ
Performance Index
0.5
0.4
0.3
0.2
0.1
0.0
**
Individual test
Group test
c739-Gal4 / +
UAS-Shi ts / +
c739-Gal4 > UAS-Shi ts
SFM not retrieved
D
Massed training
Test
33°C
25°C
24 h
α′β′
Performance Index
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
*
**
Individual test
Group test
G0050-Gal4 / +
UAS-Shi ts / +
G0050-Gal4 > UAS-Shi ts
SFM retrieved

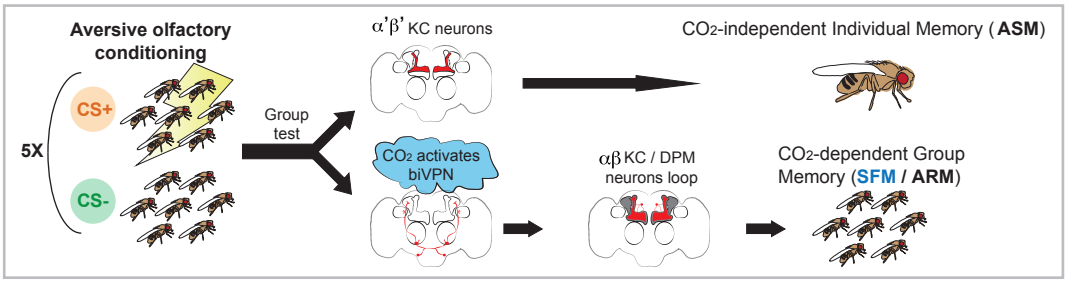


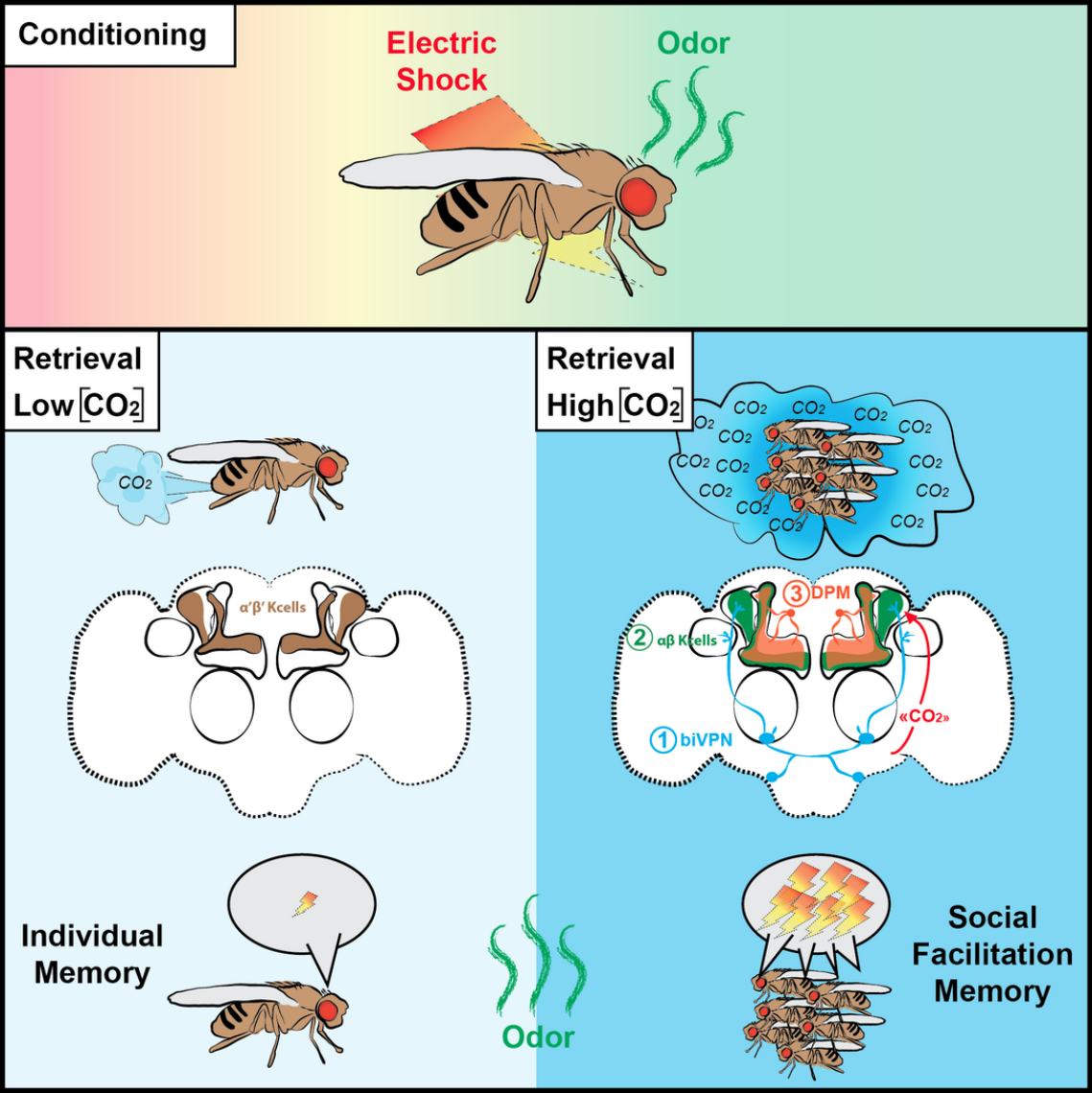


Wild Type
c739-Gal4 > UAS-Shi^{ts} (αβ)
G0050-Gal4 > UAS-Shi^{ts} (α'β')


A
αβ - Serotonin receptor
Performance Index
0.5
0.4
0.3
0.2
0.1
0.0
***
Individual test
Group test
5HT1A
c739-Gal4, Tub-Gal80ts / +
UAS-RNAi 5HT1A / +
c739-Gal4, Tub-Gal80ts > UAS-RNAi 5HT1A
B
Massed training
33°C
25°C
24 h
Test
DPM
**
VT064246-Gal4 / +
UAS-Shits / +
VT064246-Gal4 > UAS-Shits
C
DPM - Serotonin
***
5-HT
Tub-Gal80ts ; VT064246-Gal4 / +
UAS-RNAi ddc / +
Tub-Gal80ts ; VT064246-Gal4 > UAS-RNAi ddc
D
DPM - GABA
Group test
GABA
Tub-Gal80ts ; VT064246-Gal4 / +
UAS-RNAi GAD/ +
Tub-Gal80ts ; VT064246-Gal4 > UAS-RNAi GAD


A
Massed training
Test
33°C
25°C
24 h
DPM +/- CO2
Performance Index
0.5
0.4
0.3
0.2
0.1
0.0
***
*
NS
1% CO2 - + - + - +
VT064246-Gal4 / +
UAS-Shi ts/ +
VT064246-Gal4 > UAS-Shi ts
B
training
dissection
CS+ CS-
1% CO2 or air
CS+ CS-
CS+ CS-
CS+ CS-
CS+ CS-
-24hrs
-0.5hrs
-1min
T0
immediate
1min
2min
3min
CS+
Air flow
CS-
CO2
Air flow
VT064246-Gal4 > UAS-GCaMP6f
ROI1
ROI2
C
CO2 exposure
Δf(cs+)/Δf(cs-)
2.0
1.5
1.0
0.5
0.0
*
CS+
CS-
1% CO2
air
- 1min
immediate
1min
2min
3min


Aversive olfactory conditioning
5X
CS+
CS-
Group test
α'β' KC neurons
CO2 activates biVPN
αβ KC / DPM neurons loop
CO2-independent Individual Memory (ASM)
CO2-dependent Group Memory (SFM / ARM)


Conditioning
Electric Shock
Odor
Retrieval Low [CO2]
CO2
α'β' Kcells
Retrieval High [CO2]
CO2
3 DPM
2 αβ Kcells
1 biVPN
«CO2»
Individual Memory
Odor
Social Facilitation Memory